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Sequence Analysis of Measles Virus Hemagglutinin Isolated in Argentina During the 1997–1998 Outbreak

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Sequence analysis was performed on 50 measles viruses (MV) isolated in Argentina. Forty-six were obtained during the current outbreak (1997–1998), three from the previous outbreak (1991) and one sporadic case (1994). A 377-bp fragment of the hemagglutinin (H) gene was directly amplified by RT-PCR from nasopharyngeal secretions. Nucleotides 8152 to 8417 were sequenced and subjected to phylogenetic analysis. Multiple silent changes and point mutations were found in all MVs. In 1991, substitutions affected the third base in codons resulting in silent changes. In 1994 an A→C substitution at position 8321 changed amino acids 351 (Leu→Ile). In 1997–1998, an A→G substitution at position 8339 changed amino acids 357 (Val→Ile). In 3/46 viruses, guanine deletion at position 8205 changed the reading frame and insertion of an extra cytosine at nucleotide 8235 shifted it back to the original frame. Phylogenetic analysis revealed that viruses leading to the last two major outbreaks are clustered into two separate branches. MVs that prevailed until 1994 were related to genotype C1 and MVs of the current outbreak to D6. Random drift mutations rendered a 0.5 ratio of nonsilent over silent mutations in most of the MVs analyzed. However, in those showing a reading frame shift, the ratio was greater than 1, suggesting that it was driven by immune selection. ***J. Med. Virol. 60: 91–96, 2000.***

KEY WORDS: measles; genetic variability; hemagglutinin

INTRODUCTION

Measles virus (MV) is a member of the family *Paramyxoviridae,* genus *Morbillivirus* of the single-stranded negative-sense RNA viruses. MV is monotypic and stable serologically. This makes it potentially eradicable by immunization. However, genetic variability has been observed and 15 genotypes are recognized as reference strains virus [World Health Organization, 1998]; these are grouped into eight clades (A–H). Also, MV strains seem to be geographically and temporally restricted [Rima et al., 1995; Bellini and Rota, 1998]. The nonsegmented genome consists of six transcription units: nucleoprotein, phosphoprotein, matrix, fusion, polymerase, and hemagglutinin. Molecular epidemiology is focused on nucleoprotein (N) and hemagglutinin (H) genes, which contain the most variable regions at nucleotide level. The H gene spans nucleotides 7271 to 9124 and results in a glycoprotein of approximately 80 kD [Alkhatib and Briedis, 1996]. Hemagglutinin possesses at least four antigenic sites and up to nine epitopes, including a predicted B-cell epitope at amino acids 309–318 [Mäkelä et al., 1989; Hu et al., 1993]. Thirteen-conserved cysteine residues including a cysteine loop domain at amino acids 381–394 also exist in the H protein and play a critical role in folding and oligomerization of conformational antigenic epitopes. Changes in these sites known to induce neutralizing and protective antibodies may alter the immune recognition of the viral protein [Hu and Norrby, 1994; Ziegler et al., 1996].

In Argentina, despite the massive vaccination campaign in 1993 within the framework of the National Measles Control and Elimination Program, MV has reemerged with 15,000 cases reported from July 1997 to December 1998. The number of infants and children susceptible to measles has increased over time due to several factors: accumulation of unvaccinated and new annual birth cohorts; inadequate vaccination in poverty-stricken areas; and migration of children from neighboring countries with unknown measles vaccina-

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tion status. During the current outbreak in Argentina (1997–1998), 67% of reported cases are below 2 years and 83% below 5 years of age (Bilkis, personal communication), as in other developing countries [Singh and Datta, 1997]. On the other hand, in populations with high rates of coverage, measles incidence is delayed to school-aged children and adults [Outlaw and Pringle, 1995; Centers for Disease Control and Prevention, 1996].

In this study we compared MV obtained before the 1993 massive vaccination campaign and samples recovered from the current outbreak in order to assess genetic variability within circulating viral strains over a 7-year period. This is the first report attempting to characterize MV genetic diversity in our country. The GenBank accession numbers of the sequences reported in this study are AF106968 to AF107017.

MATERIALS AND METHODS

Viruses

Three viruses were obtained during the last measles outbreak (1991), 1 represents a sporadic case (1994), and forty-six were taken from the current outbreak (1997–1998). All samples were collected from nasopharyngeal secretions from nonvaccinated children aged 1–20 months. Patients were serologically confirmed as having measles. Samples were identified in the following order: country in WHO-3 letter designation, date of specimen collection by epidemic week (1–53), year and isolate number if more than one per week was obtained. New MVs were submitted to GenBank and accession numbers are given in Table I with the 15 reference strains and the external outgroup used for phylogenetic inference.

RT-PCR and Sequencing

Total RNA isolated by the guanidine-thiocyanate-phenol acid method [Chomczynski and Sacchi, 1987] was reverse-transcribed with avian myeloblastosis virus enzyme for first-strand cDNA synthesis and *Tfl* thermostable DNA polymerase for second-strand cDNA synthesis and DNA amplification by PCR. Specific outer (MH2-MH6) and inner (MH3-MH4) primers for H gene were used as described [Nakashama et al., 1995]. The 377-bp PCR product (nucleotides 8106–8482) was recovered from low-melting-point agarose gel and purified with a commercial kit according to the manufacturer's instructions. Both strands of cDNA were sequenced by direct incorporation of [γ-^{33}P]dATP end-labeled primer by the dideoxy method [Sanger et al., 1977]. Samples were processed three times in order to improve confidence in the analysis.

Phylogenetic Analysis

The sequences were aligned using ClustalW [Thompson et al., 1994]. The best tree was found using the Fitch program of Phylip (Phylogeny Inference Package, version 3.5c) software, which estimates phylogenies from distance matrix data under the additive tree model [Felsestein, 1989]. Branch lengths were calculated using the least square method [Fitch and Margoliash, 1967]. To attach statistical significance to topologies obtained by the method, randomly resampled (bootstrapped) data sets from aligned sequences were compared for a minimum of 100 data sets using the Seqboot program. Bootstrap values represent the number of times the monophyletic group consisting of the species to the right of a given node occurred during analysis. The unrooted consensus tree was drawn using the Treeview software 1.5.2.

RESULTS

Nucleotide Sequence Analysis

Comparisons were made according to Schwarz strain sequence due to the vaccination schedule applied in our country. Nucleotide numbers are referred to the complete genome [Cattaneo et al., 1989]. In 1991 only four nucleotide substitutions were found and in one sample (ARG3191) the sequence was almost identical to Schwarz strain. All substitutions affected the third position in codons. Five substitutions were detected in the single sporadic case obtained in 1994. In samples of the current outbreak, three common substitutions and occasional point mutations were present. In three viruses a guanine deletion at position 8205 and an insertion of a cytosine at position 8235 changed the reading frame for 11 amino acids (Fig. 1). The percentage of nucleotide variation, calculated as the ratio of the number of substitutions related to the reference sequence over the number of total nucleotides studied, ranged from 1.0 to 2.0. Each change in the nucleotide sequence reported above was confirmed in both strands.

According to the changes observed in sequence analysis as compared to the Schwarz vaccine strain, it was found that they could be treated as eight different sets of changes: ARG4997 with seven changes; ARG17982 with six changes; ARG1494 with five changes; and ARG2391, ARG29984, ARG22985, and ARG4097 with four different changes each. ARG3491 proved almost identical to ARG2391. All remaining viruses presented the same three changes and are represented by ARG05981. The representative member of each group of changes is underlined in Table I.

Predicted Amino Acid Sequence Analysis

The amino acid sequence was deduced for the representative member of each pattern and is shown in Figure 2, also using the Schwarz strain for comparison. No changes were expressed in ARG2391 representing the 1991 outbreak before the massive vaccination in 1993 (data not shown). In ARG1494 the nonsilent Leu→Ile change at amino acids 351 was the only one observed in comparison with 1991 MV. All MV strains circulating in the current outbreak showed a silent change (TGT→TGC) at position 300 affecting the cysteine conserved residue and the Val→Ile change at amino acid 357.

A change in the reading frame found in ARG17982, ARG4997, and ARG29984 caused variation in nine residues at position 312–322. Similar changes were

TABLE 1. MV Strains and Sequence References[a]

Strain	Description	Reference	Accession number
ARG2391	MVs/Buenos Aires.ARG/23.91	This work	AF106968
ARG3191	MVs/Buenos Aires.ARG/31.91	This work	AF106969
ARG3491	MVs/Buenos Aires.ARG/34.91	This work	AF106970
ARG1494	MVs/Buenos.Aires.ARG/14.94	This work	AF106971
ARG3097	MVs/Misiones.ARG/30.97	This work	AF106972
ARG4097	MVs/Buenos Aires.ARG/40.97	This work	AF106973
ARG4897	MVs/Buenos Aires.ARG/48.97	This work	AF106974
ARG4997	MVs/Buenos Aires.ARG/49.97	This work	AF106975
ARG5397	MVs/Buenos Aires.ARG/53.97	This work	AF106976
ARG01981	MVs/Buenos Aires.ARG/01/98/1	This work	AF106977
ARG01982	MVs/Buenos Aires.ARG/01/98/2	This work	AF106978
ARG03981	MVs/Buenos Aires.ARG/03.98/1	This work	AF106979
ARG03982	MVs/Buenos Aires.ARG/03.98/2	This work	AF106980
ARG0498	MVs/Buenos Aires.ARG/04.98	This work	AF106981
ARG05981	MVs/Buenos Aires.ARG/05.98/1	This work	AF106982
ARG05982	MVs/Buenos Aires.ARG/05.98/2	This work	AF106983
ARG05983	MVs/Buenos Aires.ARG/05.98/3	This work	AF106985
ARG0698	MVs/Buenos Aires.ARG/06.98	This work	AF106984
ARG0798	MVs/Santa Fe.ARG/07.98	This work	AF106989
ARG1398	MVs/Buenos Aires.ARG/13.98	This work	AF106986
ARG17981	MVs/Buenos Aires.ARG/17.98/1	This work	AF106987
ARG17982	MVs/Buenos Aires.ARG/17.98/2	This work	AF106988
ARG2198	MVs/Buenos Aires.ARG/21.98	This work	AF106990
ARG22981	MVs/Buenos Aires.ARG/22.98/1	This work	AF106991
ARG22982	MVs/Buenos Aires.ARG/22.98/2	This work	AF106992
ARG22983	MVs/Buenos Aires.ARG/22.98/3	This work	AF106993
ARG22984	MVs/Buenos Aires.ARG/22.98/4	This work	AF106994
ARG22985	MVs/Buenos Aires.ARG/22.98/5	This work	AF106995
ARG23981	MVs/Buenos Aires.ARG/23.98/1	This work	AF106996
ARG23982	MVs/Buenos Aires.ARG/23.98/2	This work	AF106997
ARG23983	MVs/Buenos Aires.ARG/23.98/3	This work	AF106998
ARG24981	MVs/Buenos Aires.ARG/24.98/1	This work	AF106999
ARG24982	MVs/Buenos Aires.ARG/24.98/2	This work	AF107000
ARG24983	MVs/Buenos Aires.ARG/24.98/3	This work	AF107001
ARG26981	MVs/Buenos Aires.ARG/26.98/1	This work	AF107002
ARG26982	MVs/Buenos Aires.ARG/26.98/2	This work	AF107003
ARG26983	MVs/Buenos Aires.ARG/26.98/3	This work	AF107004
ARG27981	MVs/Buenos Aires.ARG/27.98/1	This work	AF107005
ARG27982	MVs/Buenos Aires.ARG/27.98/2	This work	AF107006
ARG28981	MVs/Buenos Aires.ARG/28.98/1	This work	AF107007
ARG28982	MVs/Buenos Aires.ARG/28.98/2	This work	AF107008
ARG28983	MVs/Buenos Aires.ARG/28.98/3	This work	AF107009
ARG29981	MVs/Buenos Aires.ARG/29.98/1	This work	AF107010
ARG29982	MVs/Buenos Aires.ARG/29.98/2	This work	AF107011
ARG29983	MVs/Buenos Aires.ARG/29.98/3	This work	AF107012
ARG29984	MVs/Buenos Aires.ARG/29.98/4	This work	AF107013
ARG29985	MVs/Buenos Aires.ARG/29.98/5	This work	AF107014
ARG29986	MVs/Buenos Aires.ARG/29.98/6	This work	AF107015
ARG29987	MVs/Buenos Aires.ARG/29.98/7	This work	AF107016
ARG3098	MVs/Buenos Aires.ARG/30.98	This work	AF107017
A	Edmonston wild type/USA/1954	Rota et al. [1994]	MVO03669
B1	MVi/Younde.CAE/12.83	WHO [1998]	AF079552
B2	Mvi/Libreville.GAB/8.84	WHO [1998]	AF079551
C1	Wild Type Madrid/Spain/1994	Rima et al. [1997]	MVZ80831
C2	Mvi/JM (J. Milstein, 1977)	Rota et al. [1992]	M81898
D1	Wild Type/Bristol/England/1974	Rima et al. [1997]	MVZ80805
D2	Mvi/Johannesburg.SOA/88/4	WHO [1998]	AF085198
D3	Mvi/Chicago1	Rota et al. [1992]	M81895
D4	Mvi/Montreal.CAN/38.89	WHO [1998]	AF079554
D5	clonePAL-93H	Rota et al. [1996]	L46757
D6	Mvi/NewJersey.USA/94	Rota et al. [1996]	L46749
E	Encephalitis case/Germany/1971	Rima et al. [1997]	MVZ80797
F	SSPE case/Spain/1994	Rima et al. [1997]	MVZ80830
G	Mvi/Berkley.USA.2.83	WHO [1998]	AF079553
H	MV/China/7.93	Xu et al. [1998]	AF045201
HNT	Hamster neurotrophic/Philadelphia26	Hummel et al. [1994]	U08414
Schwarz	Schwarz vaccine	Rota et al. [1994]	U03667

[a]Characterized MVs from 1991 and 1997–1998 outbreaks were chronologically ordered by epidemiological week. Samples used for phylogenetic analysis are underlined. A–H represent the reference genotype strains. HNT was used as external outgroup and Schwarz vaccine for comparisons. The GenBank accession numbers are given.

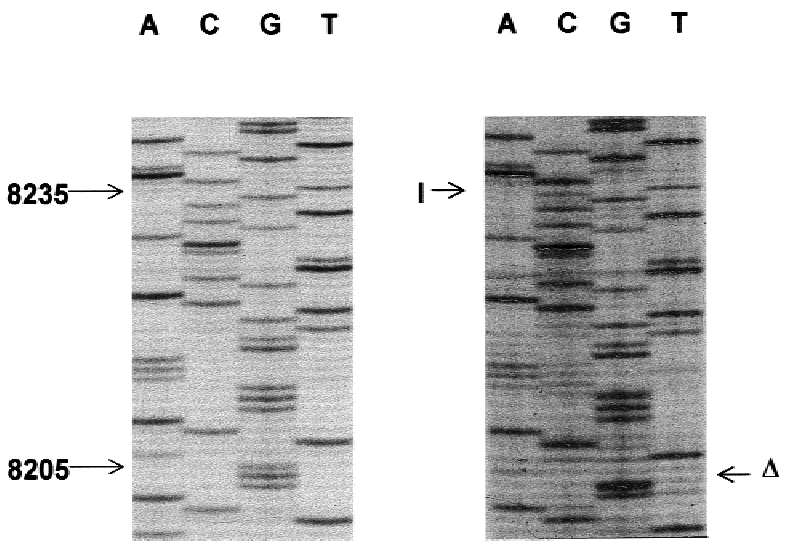

Fig. 1. Sequence gel electrophoresis comparing the Schwarz vaccine strain and ARG29984. Schwarz vaccine strain is shown on the left and ARG 29984 on the right. The guanine deletion (Δ) at position 8205 and cytosine insertion (I) at position 8235 are indicated. Same alteration is found in samples ARG4997 and ARG 17982 (data not shown).

	295	305	315	325	335	345	355	365	375
Schwarz	KLAALCHGED	SITIPYQGSG	KGVSFQLVKL	GVWKSPTDMQ	SWVPLSTDDP	VIDRLYLSSH	RGVIADNQAK	WAVPTTRTDD	KLRMETCF
ARG1494	....	....	....	....	....	......I...	....	....	....
ARG17982	....	.......DQ.	.VSASSSL..	....	....	....	..I.......	....	....
ARG4997	....	...F...DQ.	.VSASSSL..	....	....	....	..I.......	....	....
ARG4097	....	....	....	....	....	....	..I.......	....	....

Fig. 2. Amino acid predicted sequence alignment of the hemagglutinin. Numbers represent the position in the hemagglutinin protein. A dot indicates the same residue as in Schwarz vaccine strain. Nonsilent changes from the Schwarz sequence are indicated. Each strain represents a different pattern of amino acid changes.

found in ARG17982 and ARG29984. An additional Ile–Phe modification at amino acid 308 was found in ARG4997. No cysteine change at conserved positions 300, 381, and 386 was recorded.

The percentage of variation in amino acid sequence ranged from 0.01 to 0.1 due to the above change in the reading frame. Over the 7-year period the nonsilent over silent ratio was 0.5, except for MVs presenting a shift in the reading frame in which it was greater than one.

Phylogenetic Analysis

The Fitch algorithm best tree showed that viruses belonging to the last two outbreaks were located in two different branches. Before the massive 1993 vaccination campaign MV strains were clustered with the C1 reference strain and all 97–98 strains clustered with the D6 reference strain. Boostrap values were 84 for the former and 88 for the latter. Similar results were obtained using different methods (neighbor-joining and parsimony). The unrooted phenogram was plotted using Treeview 1.5.2 software (Fig. 3).

DISCUSSION

In exploring the reasons for the resurgence of measles, our laboratory genetically characterized MVs isolated from wild-type virus-infected individuals from the same outbreak. This is the first report on MV genetic characterization in Argentina.

In 1991, all substitutions found affected the third base in codons resulting in silent changes. These positions are under less selection constraint than the first and second and, as such, are more likely to change than the latter. Our results suggested that the massive 1993 vaccination campaign could have interrupted the transmission of indigenous lineage MVs. Even in the sporadic sample of 1994, taken a few months after the campaign, we found that it was related with the C1 genotype.

According to our data, MVs causing the 1991 outbreak were replaced by another strain that led to the 1997–1998 outbreak. The finding of vaccine MV in a single 1991 nasopharyngeal aspirate coincided with direct evidence of measles vaccine replication in upper respiratory tract reported by Jin et al. [1997]. Since 1997, measles resurgence has been reported in several countries of South America [Morbility and Mortality Weekly Report, 1998]. The first focus of the current outbreak began in the province of Misiones, bordering on Paraguay, with the first five cases reported in July 1997. No new cases have been reported until the appearance of a case at Buenos Aires airport. An Argentinean maid, working in an air company coming from Brazil, contracted severe measles. Later on, more than 12,000 confirmed cases spread all over Buenos Aires

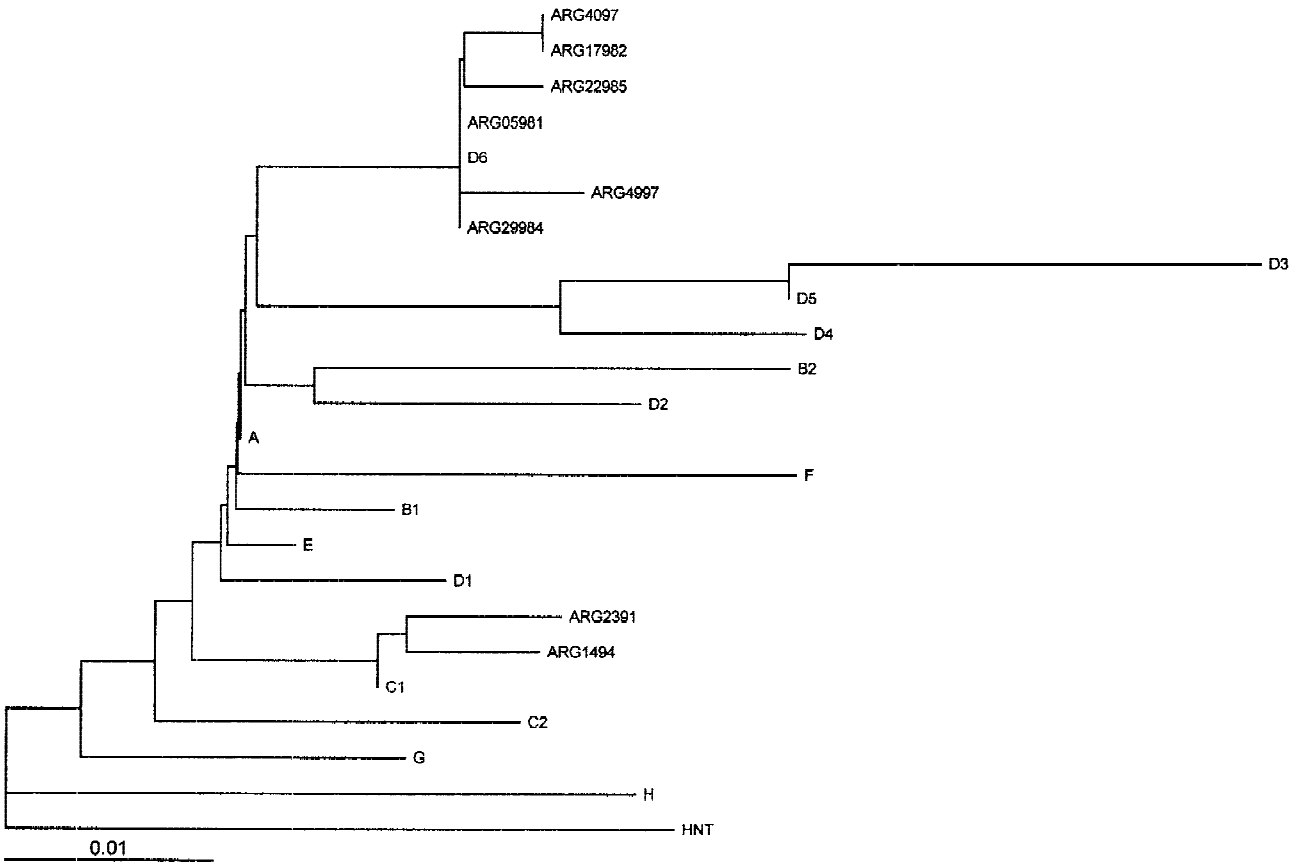

Fig. 3. Phylogenetic relationships of MV strains. The unrooted tree was generated with the Fitch algorithm of the Phylip package, version 3.5c. Strain abbreviations are described in Table I. The scale indicates 1% nucleotide divergence.

City. High rates of inner migration led to dispersion throughout the entire country. We sequenced both MV samples and results showed that they were almost the same. Identical viruses were obtained during the entire outbreak. The Val–Ile alteration at amino acid 357 was also reported in UK350/94 strains, United Kingdom's genotype I [Jin et al., 1998]. This was found circulating in Western Europe and imported to the United States [Bellini and Rota, 1998] and was called group 4 or D3. This group is now considered as genotype D6. Further characterization of viruses in neighboring countries would contribute to clarify the origin of MV in ours. In all likelihood, the virus encountered a poorly protected population and remained as indigenous.

Phylogenetic analyses are usually based on the hypervariable region of the N gene located in the carboxyl terminus [Taylor et al., 1991]. However, these changes cannot significantly alter its product without losing the ability of replication. Grouping of MV strains proves the same whether N or H genes are compared [Rima et al., 1997]. We analyzed the middle portion of the H gene because hemagglutinin is a structural component of the virion envelope that stimulates high titers of neutralizing antibodies and plays a major role in initial stages of viral infection. Many biological functions are exterted by this protein: attachment to susceptible host cells by binding to the extracellular CD46 domain, a receptor for MV [Hsu et al., 1998]; and as an epitope for both cytotoxic T-lymphocyte [Jaye et al., 1998] and B-cells [Mäkelä et al., 1989]. Changes in this protein may alter its biological functions [Ziegler et al., 1996]. In addition, biased hypermutation has been reported for this portion of the H gene [Cattaneo et al., 1989].

The H gene region amplified in our study contained a B-cell epitope at residues 309–318. The change in the reading frame affected either polarity or charges in 3/9 residues within this epitope. Samples carrying this alteration were detected throughout the current outbreak without epidemiological links.

Several changes have been reported for the H gene sequence, and genetic and antigenic changes may have been accumulated in the last years [Tamin et al., 1994]. In the Coventry area in the U.K., an early termination signal at amino acid position 583 shortened the H protein by 35 amino acid residues [Outlaw and Pringle, 1995]. Changes in the reading frame have also been described for variants selected by monoclonal antibodies [Hu et al., 1993]. As the change in the reading frame described in our results does not alter the length of the analyzed fragment, nothing could be said about biological properties of the entire H protein before a deeper analysis.

In some cases, quasispecies variation and possibly PCR misincorporations caused more than one band at

the same position. As the sequence reaction was performed three times for both strands, a consensus sequence was assumed and analyzed. The existence of fluctuations in the MV population could not be discarded.

The direct sequencing method is useful for countries with large territories where viral viability preservation is troublesome. In well-preserved samples (18/50), MVs could be isolated in B95a cells with significant CPE. These isolations were confirmed by IFA and PCR but not used for sequencing. We sequenced the amplicons following RT-PCR directly from clinical samples, thus minimizing possible errors in RT-PCR and changes due to adaptation to primate cells as Vero and B95a [Rima et al., 1997]. Although one of the isolated samples had the reported change in the reading frame, the fact of having this strain growing in cell culture suggests that the deletion-insertion event do not affected the viral viability. However, the biological implications of these changes are still poorly understood. Characterization of hemagglutinin proves effective to classify circulating MV strains. Further research might be done to elucidate viral mechanisms of immune response evasion.

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